The matrix of the constructs had resultant matrix of type II collagen and proteogrycan which confirmed histochemically. The constructs formed *via* cell condensation at 10^7cells/cm^2 grew in thickness until the day 14 of P1 culture. They grew in size until the day 28 of P1 culture.
The tissues were stained strongly with safraninO, and there were partially columnar pattern. Immunohistochemical analysis revealed that typeIIcollagen was abundantly deposited in the tissue. Moreover with toluidine blue staining, there was a metachromatic matrix. Interestingly, LIPUS group was also more strongly stained than sham group by histology.
In vivo study, histology of repair tissue with the chondrocyte plate in 2wks was not significant different about LIPUS group and non-treated LIPUS (sham) group. But the treated by the plate group, LIPUS and sham group, were repaired better than control group by histology.
Conclusions: The chondrocytes of P1 cells prepared at 10^7cells/cm^2 developed into the scaffold-free chondrocyte plate which was similar to native cartilage under existing cell-cell interactions.
As a result for application of LIPUS, we could stimulate the matrix synthesis of the scaffold-free chondrocytes plate in vitro study.
In vivo study, we didn't show the effect of LIPUS to stimulate the matrix synthesis of the plate.

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DECREASED CHONDROCYTE HYPERTROPHIC DIFFERENTIATION BY INHIBITION OF CYCLOOXYGENASE-2

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Purpose: Chondrocyte hypertrophic differentiation is an essential process during endochondral bone formation, however it hampers the application of many cartilage regenerative techniques and may play a role at the onset of osteoarthritis. Clinically, heterotopic bone formation after orthopaedic surgery is suppressed by non-steroidal anti-inflammatory drugs (NSAIDs) and recent studies point to an essential role of Cyclooxygenase-2 (COX-2) in osteocytes during endochondral ossification. COX-2 and its metabolite PGE_2 are thought to regulate the function of bone morphogenic protein-2 (BMP-2) and vice versa, which might provide an explanation for the role of COX-2 during endochondral ossification.
In this study, we aim to determine the involvement of COX-2 in chondrocyte hypertrophy and provide an explanation for the suppressive effect of NSAIDs on heterotopic ossification and fracture healing.
Methods: To study the role of COX-2 during endochondral ossification, ATDC5 cells were differentiated in the chondrogenic lineage in the presence of BMP-2 for 14 days. The NSAIDs indomethacin (COX-1/COX-2) or NS398 (COX-2 specific) were used to inhibit COX-activity. Expression analysis was performed by RT-qPCR and western blot (Collagen II, Collagen X, RunX2, COX-2, BMP-2). Growth curves were obtained from increasing indomethacin- and NS398-concentrations. Cell numbers were determined by X-violet staining. Rabbit periosteal grafts were differentiated for 3 weeks in an agarose sandwich in the presence of BMP-2 (30 ng/nl) and indomethacin (2 μM) or NS398 (2 μM). Differentiated grafts were harvested for RT-qPCR and (immuno-)histological analysis. PGE_2 production in medium was measured using a specific ELISA.
Results: Hypertrophic differentiation of ATDC5 cells was obvious from day 14. Chondrogenesis was accompanied by upregulation of COX-2 and addition of BMP2 (30 ng/ml) lead to increased upregulation of COX-2 expression and PGE_2 in the media. Accordingly, expression of Collagen X was also significantly upregulated. COX-2 activity was completely inhibited by indomethacin or NS398. Surprisingly, specific inhibition of COX-2 by NS398 did not affect Collagen II expression whereas expression of RunX2 and Collagen X was significantly decreased in a concentration dependent way. COX-inhibition by indomethacin showed similar results, however had inhibitory effects on Collagen II expression at higher concentrations. Expression of BMP-2 decreased upon increasing NSAID concentrations, confirming a functional connection between COX-2 and BMP-2. To determine whether COX-inhibition specifically affects differentiation, proliferation rate of ATDC5 cells under proliferating and differentiating conditions was determined. No significant effect on proliferation was detected. Chondrocyte hypertrophy of periosteal grafts was dependent on BMP-2. This BMP-2 mediated hypertrophy was suppressed by COX-inhibition as determined by ELISA, RT-qPCR and histological analysis, confirming our findings in ATDC5 cells.
Conclusions: Our data show that COX-2 is upregulated during chondrogenesis and that BMP-2 induces chondrocyte hypertrophy and COX-2 expression, suggesting a functional connection. Using two independent systems we were able to show that inhibition of COX-activity decreased chondrocyte hypertrophy. Insight into the mechanism by which COX-2 influences endochondral ossification is essential to understand the effect of NSAIDs on fracture healing. Our data may provide a novel strategy to improve the outcome of cartilage regenerative medicine.

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EXPRESSION OF MESENCHYMAL STEM CELL MARKERS IN SYNOVIAL MEMBRANES AND OSTEOARTHRITIC CARTILAGE REPAIR

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Purpose: Mesenchymal stem cells (MSCs) have been identified in human synovial membrane and articular cartilage from patients with osteoarthritis (OA), but detailed quantification studies of MSCs were not performed. The purpose of this study was to quantitatively measure the expression of MSC markers in synovial membranes from human OA (OA) and health joints.
Methods: Synovial membrane-derived cells were isolated from health and OA joints and characterized by flow cytometry for hematopoietic (CD34 and CD45) and mesenchymal (CD44, CD29, CD73, CD90, CD105 and CD271) markers. Chondrogenesis was assessed by staining for proteoglycans and type II collagen, adipogenesis by using a stain for lipid drops and osteogenesis by detecting calcium deposits. The co-expression of CD44, CD90, CD105 and CD271 was determined by immunofluorescence in OA and normal synovial membranes and spontaneous cartilage repair.
Results: More than 90% of OA synovial membrane-derived cells were positive for CD44, CD73, and CD90 and negative for CD34 and CD45. OA synovial membrane-derived cells were also positive for CD29 (85.23%), CD117 (72.35%) and CD105 (45.5%). Micropellet analyses showed that culture of the cells with TGF-beta3 stimulated proteoglycan and type II collagen synthesis. Synovial membrane-derived cells culture developed lipid droplets in adipogenic medium and calcification in oteogenic medium. Cells positive for MSC markers were diffusely distributed in OA synovial membranes. In health synovia, these cells were localized in the subintimal zone. The number of cells expressing MSCs markers was higher in OA synovial membranes than in synovia from nor-

mal joints (6.1% vs. 2.2%), corresponding the highest percentage of cellular expression to the co-expression of CD90/CD271 (9.8% vs. 2.6%). Spontaneous repair tissue contained cells positive for CD44 (12.3%), CD90 (10.4%) and CD271 (9.9%) antigens, but the CD105 antigen was absent.
Conclusions: These results suggest that during the OA process an increment of MSC percentage occur in synovial membrane. Absent expression of the CD105 antigen in the OA cartilage repair indicates that cells expressing this marker may be necessary for effective repair processes in OA cartilage.
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EXPRESSION ANALYSIS OF mRNA DURING DIFFERENTIATION FROM MSCS TO MATURE CHONDROCYTES

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Purpose: Mesenchymal stem cells (MSCs) are considered as a potential cell therapy to treat a variety of rheumatic diseases. MSCs are widely distributed in the body, including bone marrow (BM). Chondrogenic differentiation process involves the differentiation of MSCs towards cartilage or bone. Cartilage tissue engineering uses efficient protocols that suppress differentiation towards hypertrophic chondrocytes, providing a useful in vitro model for studying the gene regulation of chondrogenesis and cartilage development. This model may provide new molecular targets for rheumatic diseases treatment. The aim of this study was investigate the transcriptional regulation of genes implicated in the different steps of chondrogenesis in normoxia versus hypoxia conditions.
Methods: MSCs, isolated from 5 BM were expanded in monolayer. CD105+ and CD105- cells were separated from total BM by MACS Technology (Miltenyi Biotec, Spain) and their phenotype were characterized by flow cytometry. The three populations of MSCs (total BM, CD105+ and CD105- BM) were subjected to chondrogenesis by means of high-density 3D aggregate culture in normoxia and hypoxia conditions. Chondrogenesis was studied at different intervals of time: 0, 2, 4, 7, 14, 21, and 28 days and differentiation was confirmed by histochemistry (hematoxylin-eosin, Masson's thrichome, alcian blue, safranin O), immunohistochemistry (types II and I collagens, and aggrecan) and quantitative real Time PCR (qPCR) techniques. For qPCR experiments RNAs were isolated using Trizol reagent (Invitrogen, Spain). Different primers were designed to study mRNA expression of different genes implicated in the different stages of chondrogenesis (MSCs, condensation & chondroprogenitors, chondrocytes and prehypertrophic chondrocytes).These primers included Sox2, Nanog, OCT3/4, CDH2, TNC, Sox9, Agg, Col I, Col II, Col X and RunX2.
Results: At the starting point of the experiments, the stem cell phenotype markers (SOX2, Nanog and OCT3/4) showed higher expression levels in the CD105+ population. At 14-28 days in hypoxia and at 21-28 days in normoxia the expression levels of Nanog and OCT3/4 showed to be higher in the total bone marrow and CD105- populations. Instead, the level of SOX2 at the same times of the experiment showed to be higher in the CD105+ population. These markers remained expressed until day 28, both in normoxia and in hypoxia, indicating that not all the stem cells were differentiated towards chondrocytes but some of them remained in a state of indifferentiation. With regard to cartilage specific markers, the CD105- population showed increased levels of SOX9 and AGG mRNA than CD105 + and total bone marrow ones, however the latter showed the highest expression levels of Col II followed by CD105- and CD105 + populations. In this way, the overexpression of Col II in hypoxia was accentuated between 7 and 21 days while in normoxia took place from 14 to 28 days.
Conclusions: Hypoxia accelerates the chondrogenic differentiation process from bone marrow-derived adult mesenchymal stem cells to mature chondrocytes. Isolation of CD105 + and CD105- populations shows no improvement in chondrogenesis with regard to the total bone marrow population.In addition, while the CD105+ population seems to differentiate in the experimental conditions, the CD105- one shows to remain in an indifferentiated state, maintening an important expression of SOX2, NANOG and OCT3/4 genes.
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AN ORGAN CULTURE MODEL TO STUDY BIOLOGICAL REPAIR OF THE DEGENERATE INTERVERTEBRAL DISC

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The current surgical procedures used to treat the degenerate intervertebral disc (IVD) are inadequate. Vertebral fusion can frequently reduce back pain, but does not preserve motion segmental mobility and thus may lead to subsequent degeneration in adjacent motion segments. In contrast, artificial disc prostheses preserve mobility, but in the long-term may result in serious complications due to wear, loosening, subsidence or mechanical failure. Biological repair of the degenerate IVD could provide a means of overcoming these problems. In vitro organ culture models using large IVDs have recently been described for this purpose, but they have suffered from the inability to achieve long-term NP cell viability, due to impaired nutrient supply because of endplate calcification and the postmortem formation of blood clots blocking the trans-endplate nutrition path.
Purpose: To develop a bovine coccygeal IVD organ culture model of disc degeneration with long-term cell survival, in which induced biological repair can be studied.
Methods: IVDs were isolated from bovine tails by parallel cuts through the adjacent vertebral bodies, and remaining bone and most of the cartilage endplates were removed using a high-speed bone burr. The explants were maintained in culture in DMEM, supplemented with 5% FBS and antibiotics, with medium changed twice weekly. Degeneration was induced by injecting trypsin into the center of the NP. Cell viability was monitored in a 1 mm slice through the center of the disc, using a live/dead fluorescence assay (Live/Dead®, Invitrogen) and confocal microscopy. Tissue morphology was examined by histology. Annulus fibrosus (AF) and nucleus pulposus (NP) were extracted using 15 volumes 4M GuHCl containing protease inhibitors, and changes in the abundance and degradation of chondroadherin and fibromodulin were analysed by immunoblotting. GAG content was measured using the DMMB assay.
Results: Intact bovine discs maintained in free swelling culture with no external load applied demonstrated cell viability for up to 6 weeks. The amount of GAG retained in the tissue decreased slowly during the culture period and cell clustering reminiscent of cartilage and disc degeneration was found. As it is not practical to wait for 4-6 weeks before repair potential of various treatments